High mobility group box 1 induces calcification of aortic valve interstitial cells via toll-like receptor 4

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Abstract. Chronic inflammation and the calcification of aortic valve interstitial cells (AVICs) are the primary etiologies of calcific aortic valve disease (CAVD). However, the underlying mechanism remains to be elucidated. The present study investigated the importance of high mobility group box 1 (HMGB1) via toll-like receptor 4 (TLR4) for the regulation of inflammation and calcification in AVICs. It was determined that the expression levels of HMGB1 and TLR4 were increased in the calcific region of aortic valves with CAVD. In cultured primary AVICs from wild-type mice, HMGB1 treatment demonstrated a dose-dependent increase in mineralization levels and osteogenic gene expression. These effects were significantly reduced in AVICs obtained from TLR4 knockout mice (TLR4$^{-/-}$). In addition, calcification was inhibited by TLR4-specific antibodies in primary AVICs. HMGB1 induced the activation of p38 and nuclear factor-κB (NF-κB) in TLR4$^{-/-}$ primary AVICs, and inhibited p38 and NF-κB in wild-type AVICs treated with TLR4-specific antibodies. The present study demonstrated that TLR4 may function as an essential mediator of HMGB1-induced calcification and in the activation of p38 and NF-κB.

Introduction

Calcific aortic valve disease (CAVD) is a slow progressive pathologic process that exhibits mild thickening of the aortic valve and calcification of valve leaflets (1). CAVD may be an actively regulated process that includes chronic inflammation, lipoprotein deposition, the renin-angiotensin system, extracellular matrix remodeling, the activation of specific osteogenic signaling pathways and apoptosis. However, degeneration of the aortic valve is the key etiology of CAVD (occurring in 81.9% of CAVD patients) (2), and specific osteogenic signaling pathways determine the activation and differentiation of the resident fibroblasts or quiescent valve interstitial cells (VICs) into myofibroblasts (activated VICs) and osteoblast-like cells (osteoblastic VICs) with subsequent micro- and macro-calcification (3-5). Therefore, inflammatory responses participate in all stages of CAVD and promote its pathophysiological progression (6,7).

High mobility group box 1 (HMGB1) is a non-histone DNA binding protein (8). HMGB1 is involved in the stabilization of DNA and promotion of gene transcription (9). However, previous studies have demonstrated that HMGB1 participates in inflammation through effects on macrophages (10,11). The expression level of HMGB1 was demonstrated to be increased in acute and chronic inflammatory diseases, such as type 2 diabetes (12), chronic asthma (13) and chronic rhinosinusitis (14). HMGB1 signals through receptor of advanced glycation end products, toll-like receptor (TLR) 2 and TLR4, thereby stimulating an inflammatory response in cells (15). However, the role of HMGB1 in CAVD remains to be elucidated.

A previous study demonstrated that TLR4 proteins are highly expressed in human aortic valve leaflets and aortic valve interstitial cells (AVICs) (16). Stimulation with LPS may result in the activation of the nuclear factor-κB (NF-κB) signaling pathway (17). The TLR4 signaling pathway has been demonstrated to enhance the pro-osteogenic response in AVICs and may promote the progression of CAVD (18).

Chronic inflammation is crucial for the development of CAVD, as it was observed to promote the osteoblastic differentiation of AVICs (19). The present study used immunohistochemistry to demonstrate that HMGB1 and TLR4 were expressed in the aortic valve of CAVD samples. In addition, it was revealed that HMGB1 is important for osteoblastic differentiation in AVICs, which is mediated by TLR4. TLR4 was demonstrated to be essential for HMGB1-induced runt-related transcription factor 2 (Runx2), msh homeobox 2 (Msx2), bone morphogenetic protein 2 (BMP2) and osteopontin (OPN) expression in AVICs. These findings provide a novel mechanism for CAVD progression and improve the understanding of aortic valve disease.

Materials and methods

Reagents and antibodies. HMGB1 (R&D Systems, Inc., Minneapolis, MN, USA) was used to stimulate AVICs. The

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antibodies used to detect HMGB1 (catalog no. ab18256) and TLR4 (catalog no. ab13556) by immunohistochemical analysis were purchased from Abcam (Cambridge, UK). The 3,3'-Diaminobenzidine Liquid Substrate system, Alkaline Phosphatase Diethanolamine Activity kit and alizarin red S stain were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM): F12, supplemented with 20% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/l streptomycin, was obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The primary antibodies used were specific to Runx2 (catalog no. ab23981), Msx2 (catalog no. ab69058), BMP2 (catalog no. ab14933), OPN (catalog no. ab91655; Abcam), β-actin (catalog no. 4970S; Cell Signaling Technology, Inc., Danvers, MA, USA), p38 mitogen-activated protein kinase (MAPK; catalog no. 8690S; Cell Signaling Technology, Inc.), phosphorylated (p)-p38-MAPK (p-p38-MAPK; catalog no. 9215S; Cell Signaling Tecnhology, Inc.), NF-κB (catalog no. 8242S; Cell Signaling Technology, Inc.) and p-NF-κB (catalog no. 3033S; Cell Signaling Technology, Inc.). SignalStain® Boost Detection reagent (catalog no. 8114; Cell Signaling Technology, Inc.) was used to detect primary antibodies. Activation of TLR4 was inhibited using an anti-mouse TLR4/MD-2 antibody (cat. no. 16-9924; eBioscience, Inc., San Diego, CA, USA), and a mouse IgG isotype control antibody (catalog no. 16-4714; eBioscience, Inc.) was used as a negative control.

Clinical samples. Human aortic valves with calcific regions were obtained from 3 patients who underwent a valve replacement surgery. Normal aortic valve leaflets were collected from the explanted hearts of 3 patients undergoing heart transplantation surgery. All patients were admitted to The Affiliated Tongji Hospital (Shanghai, China) between August 2015 and December 2016. All patients were male (weight, 65±5 kg; age, 70±3 years). The protocols of the present study were approved by the Ethics Committee of Tongji Hospital, Shanghai Tongji University School of Medicine (Shanghai, China) and written informed consent was obtained from all patients.

Primary AVIC culture. Male, 6-week-old (weight, 10-15 g) wild-type (C57BL/6; n=20) and TLR4 knockout mice (TLR4$^{-/-}$; n=20) were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China). To avoid the stress response, the animals had free access to water and a normal diet for 2 weeks and housed at 5 mice per cage, under a 12-h light/dark cycle at room temperature. The mice were anesthesized with 100 mg/kg sodium pentobarbital and their aortic valves were obtained for the culture of primary AVICs using the methods described previously (20). Valve leaflets were subjected to collagenase digestion and gently scraped to expose the endothelial layer. The leaflets were then divided into 1-2 mm^2 pieces and cultured in DMEM: F12 (1:1) supplemented with 20% FBS, 2 mmol/l L-glutamine (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/l streptomycin. The cells were cultured in 5% CO$_2$ at 37˚C. When 80% confluence was reached, AVICs were passaged using 0.25% trypsin-EDTA. AVICs at passages 3 and 8 were used for further experiments. The animal experimental procedures employed in the current study complied with the Animal Management Rules of the Chinese Ministry of Health (Document no. 55, 2001), and were approved by the Animal Care Committee of Tongji University (Shanghai, China).

Immunohistochemistry. Human calcific (n=3) and non-calcific aortic valves (n=3) were used for histological and immunochemical analysis. The samples were fixed in 4% paraformaldehyde overnight and divided into serial 5-μm cryosections. The sections were stained with 5% hematoxylin and 0.5% eosin for 15 min at room temperature. Immunohistochemical analysis was performed using anti-HMGB1 (dilution, 1:50) and anti-TLR4 (dilution, 1:50) antibodies at room temperature for 2 h. Following incubation with HRP-conjugated secondary antibodies (dilution, 1:100) at room temperature for 1 h, the sections were incubated with 3,3'-diaminobenzidine (catalog no. D8001; Sigma-Aldrich; Merck Millipore). The expression levels of HMGB1 and TLR4 detected by immunohistochemistry were determined using Image Pro-Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). The results were determined as integrated optical density/area.

In vitro calcification of AVICs. Primary AVICs were isolated from the aortic valves of C57BL/6 and TLR4$^{-/-}$ knockout mice. AVIC calcification was induced in an osteogenic medium containing DMEM, supplemented with 15% FBS, 50 mg/ml ascorbate-2-phosphate, 10 nM dexamethasone and 10 mM β-glycerol phosphate (all purchased from Gibco; Thermo Fisher Scientific) as described previously (21). The culture medium was refreshed every 48-72 h, and the cells were harvested after 3 weeks. The extent of calcification of AVICs was determined by alizarin red S staining. The cells were washed in distilled water and then exposed to freshly prepared 2% alizarin red S (pH 4.1-4.3) for 5 min, where a red/orange color indicated positive staining. For quantitative analysis of alizarin red S staining, the dye was released from the cell matrix by incubating the cells with cetylpyridinium chloride for 15 min at room temperature. The quantity of released dye was determined using a spectrophotometer at 540 nm. Alkaline phosphatase (ALP) activity was determined using spectrophotometry to quantify the level of the p-nitrophenol in AVICs, according to the methods described previously (22). The quantity of alizarin red S staining and ALP activity were normalized to the total quantity of cellular protein using Image Pro-Plus software version 6.0. The quantity of cellular protein was measured by Bichinchoninic Acid assay.

Inhibition of TLR4 activation. Primary AVICs from C57BL/6 mice were seeded onto 6-well plates at a density of 5x10^6 cells/well, before they were pretreated with 5 μg/ml anti-TLR4 antibody (1:20) or 5 μg/ml mouse IgG (1:20) as a negative control for 1 h, and subsequently stimulated with HMGB1 at concentrations of 0.5, 1 or 2 μg/ml for 60 min or 3 weeks.

Western blotting. AVICs (5x10^6 cells) were lysed with the ProteoJET Mammalian Cell Lysis reagent (Fermentas; Thermo Fisher Scientific, Inc.) to extract cytoplasmic proteins. Equal quantities of protein (50 μg) were subjected to 10% SDS-PAGE and blotted onto polyvinylidene difluoride membranes. The membrane was blocked with 5% bovine

serum albumin (catalog no. V900933; Sigma-Aldrich; Merck Millipore) and probed with antibodies against TLR4 (dilution, 1:500), Runx2 (dilution, 1:1000), Msx2 (dilution, 1:1,000), BMP2 (dilution, 1:1,000), OPN (dilution, 1:1,000), β-actin (dilution, 1:2,000), p38-MAPK (dilution, 1:1,000), p-p38-MAPK (dilution, 1:1,000), NF-κB (dilution, 1:1,000) and p-NF-κB (dilution, 1:1,000) overnight at 4°C, followed by incubation with HRP-conjugated secondary antibodies (dilution, 1:5,000) for 1 h at room temperature. The blots were developed using an enhanced chemiluminescence detection system (Merck Millipore). Images were captured, and the intensity of each band was analyzed with Quantity One software version 4.62 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data are expressed as the mean ± standard deviation. Student's paired *t*-test was performed to compare paired samples and analysis of variance was used for multiple group comparisons, followed by Friedman's *post-hoc* test. P<0.05 was considered to indicate statistically significant difference. Statistical analyses were performed using SPSS software version 20 (IBM SPSS, Armonk, NY, USA).

Results

Calcific aortic valves exhibit HMGB1 and TLR4 expression. To investigate the pathology of calcific aortic valves, calcific (n=3) and non-calcific aortic valves (n=3) were stained with HE. Routine pathological examination of calcific aortic valves revealed calcification, macrophage rupture and AVIC thickening close to the region of calcification (Fig. 1A). In calcific aortic valves, the positive expression of HMGB1 and TLR4 was detected across the region of calcification and the integrated optical density/area level of HMGB1 and TLR4 was significantly higher when compared with those in the control aortic valve group (HMGB1, 78.1±7.6 and TLR4, 84.2±7.3 in calcific aortic valves vs. HMGB1, 25.1±3.4 and TLR4, 28.0±2.4 in non-calcific aortic valves; P<0.01; Fig. 1B). These results indicated that HMGB1 and TLR4 may contribute to the calcification of the aortic valves.

HMGB1 induces AVIC calcification. The present study investigated whether HMGB1 was involved in the calcification of AVICs in mice. Primary AVICs were stimulated with HMGB1 in order to assess its effects on the calcification of AVICs and the expression levels of Runx2, Msx2, BMP2 and OPN. The ALP activity and alizarin red S staining assays demonstrated that HMGB1 treatment promoted calcification in a dose-dependent manner (Fig. 2A and B). The levels of ALP activity and alizarin red S staining increased significantly at concentrations of 1 and 2 μg/ml HMGB1 when compared with the control group (ALP activity, 1 μg/ml HMGB1, 434.32±34.51 vs. 285.33±34.95, P<0.01 and 2 μg/ml HMGB1, 510.34±29.28 vs. 285.33±34.95, P<0.01; alizarin red S staining assay, 1 μg/ml HMGB1, 148.21±7.43 vs. 114.33±17.50, P<0.05 and 2 μg/ml HMGB1, 174.67±9.03 vs. 114.33±17.50, P<0.01; Fig. 2A and B). Runx2, Msx2, BMP2 and OPN have been previously demonstrated to be involved in and promote the calcification of AVICs (23). The present study used western blotting to determine the effects of HMGB1 on their protein expression levels. Following exposure of AVICs to HMGB1,
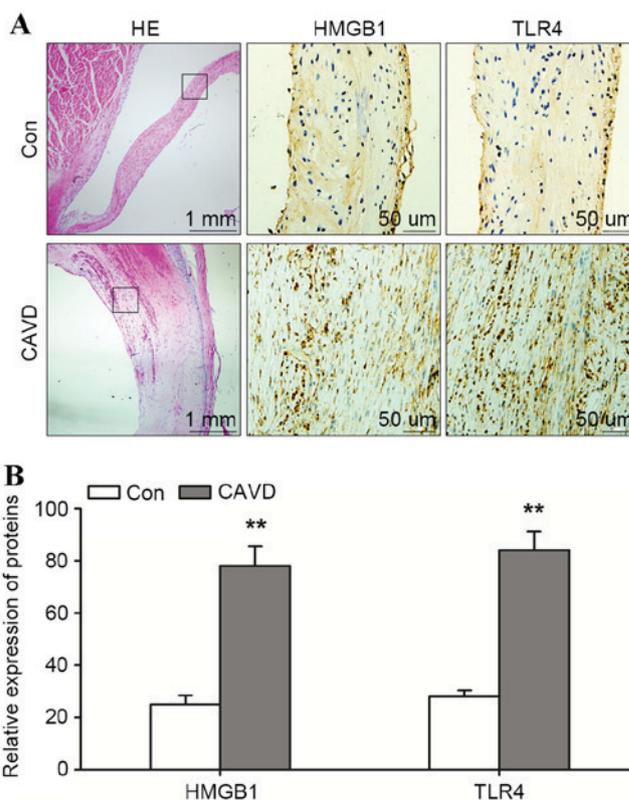
Figure 1. Expression of HMGB1 and TLR4 in the calcification region of CAVD arterial valves. Human aortic valves with calcific and normal aortic valve leaflets were collected from explanted hearts of patients and were assessed by histological and IHC analyses. (A) Sections were stained with HE or with specific antibodies against HMGB1 and TLR4. The black boxes represent the area presented in the IHC staining images. (B) The expression levels of HMGB1 and TLR4 were detected by IHC and were quantified using Image Pro-Plus version 6.0. Data are presented as integrated optical density/area. Three different sections and five different fields of view were analyzed for each section (n=3). **P<0.01 vs. Con. HMGB1, high mobility group box 1; TLR4, toll-like receptor 4; CAVD, calcific aortic valve disease; IHC, immunohistochemistry; HE, hematoxylin and eosin; Con, control.

the protein expression levels of Runx2, Msx2, BMP2 and OPN demonstrated a dose-dependent increase (Fig. 2C). High doses (1 and 2 μg/ml) of HMGB1 significantly induced the expression of these genes (P<0.01; Fig. 2D). These results indicated that, HMGB1 upregulated calcification and the expression of associated genes in primary AVICs in a dose-dependent manner, which suggests that HMGB1 may contribute to the calcification process in AVICs.

TLR4 mediates HMGB1-induced calcification of AVICs. In order to determine whether TLR4 mediated the HMGB1-induced calcification in AVICs, as well as the expression levels of associated genes in AVICs, the calcification and protein expression levels of Runx2, Msx2, BMP2 and OPN following HMGB1 treatment in primary AVICs derived from the valves of wild-type (C57BL/6) or TLR4^{-/-} mice, were compared. A TLR4 assay was used in order to identify the phenotype of wild-type and TLR4^{-/-}. TLR4 protein expression was detected in the wild-type, but not in TLR4^{-/-}-derived AVICs (Fig. 3A). As 2 μg/ml HMGB1 induced the highest calcification in AVICs (Fig. 2A and B), this concentration was used for further experiments. Primary AVICs were
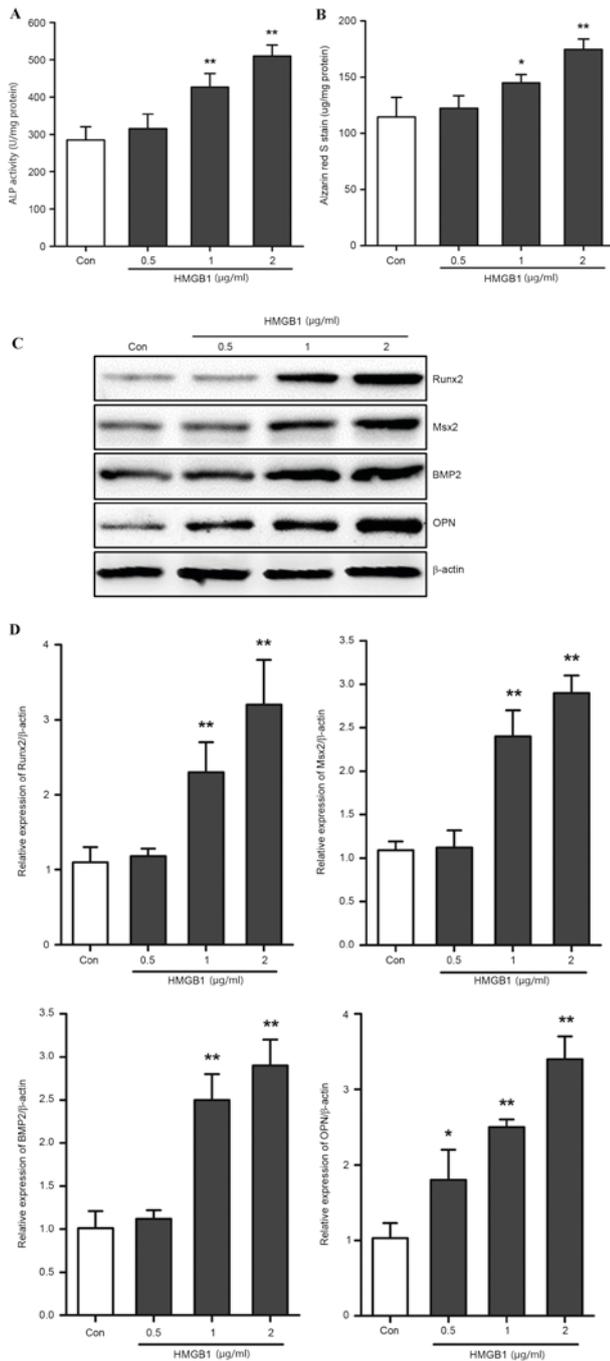
Figure 2. HMGB1 induced calcification of AVICs. Primary AVICs were incubated with 0.5, 1 and 2 µg/ml HMGB1 for 3 weeks. Quantification of (A) ALP activity and (B) alizarin red S staining in AVICs. (C) Western blotting was used to determine the protein expression levels of Runx2, Msx2, BMP2 and OPN. (D) Quantification of band densities by densitometry in 3 independent experiments relative to β-actin (n=3). *P<0.05 and **P<0.01 vs. Con. HMGB1, high mobility group box 1; AVICs, aortic valve interstitial cells; ALP, alkaline phosphatase; Runx2, runt-related transcription factor 2; Msx2, msh homeobox 2; BMP2, bone morphogenetic protein 2; OPN, osteopontin; Con, control.

Figure 3. TLR4 mediates HMGB1-induced calcification in AVICs. Primary AVICs were derived from TLR4⁻/⁻ and C57BL/6 mice. They were subsequently pretreated with a TLR4-specific antibody, and a mouse IgG was used as a control. (A) TLR4 expression in TLR4⁻/⁻ and C57BL/6-derived AVICs was determined using western blotting (n=4 per genotype). Quantification of (B) ALP activity and (C) alizarin red S staining. (D) Western blotting was used to determine the protein expression levels. (E) Quantification of western blotting by densitometry in 3 independent experiments relative to β-actin (n=3). **P<0.01 vs. C57BL/6 group; ##P<0.01 vs. the mouse IgG treatment group. TLR4, toll-like receptor 4; HMGB1, high mobility group box 1; AVICs, aortic valve interstitial cells; ALP, alkaline phosphatase; Runx 2, runt-related transcription factor 2; Msx2, msh homeobox 2; BMP2, bone morphogenetic protein 2; OPN, osteopontin.

incubated with 2 µg/ml HMGB1 for 3 weeks. TLR4⁻/⁻ AVICs exhibited a significant reduction in ALP activity and alizarin red S staining when compared with wild-type AVICs (P<0.01; Fig. 3B and C). In addition, the protein expression levels of Runx2, Msx2, BMP2 and OPN, were significantly reduced in TLR4⁻/⁻-derived AVICs when compared with the wild-type

AVICs (P<0.01; Fig. 3D and E), suggesting that TLR4 may be important for HMGB1-mediated calcification of AVICs. The
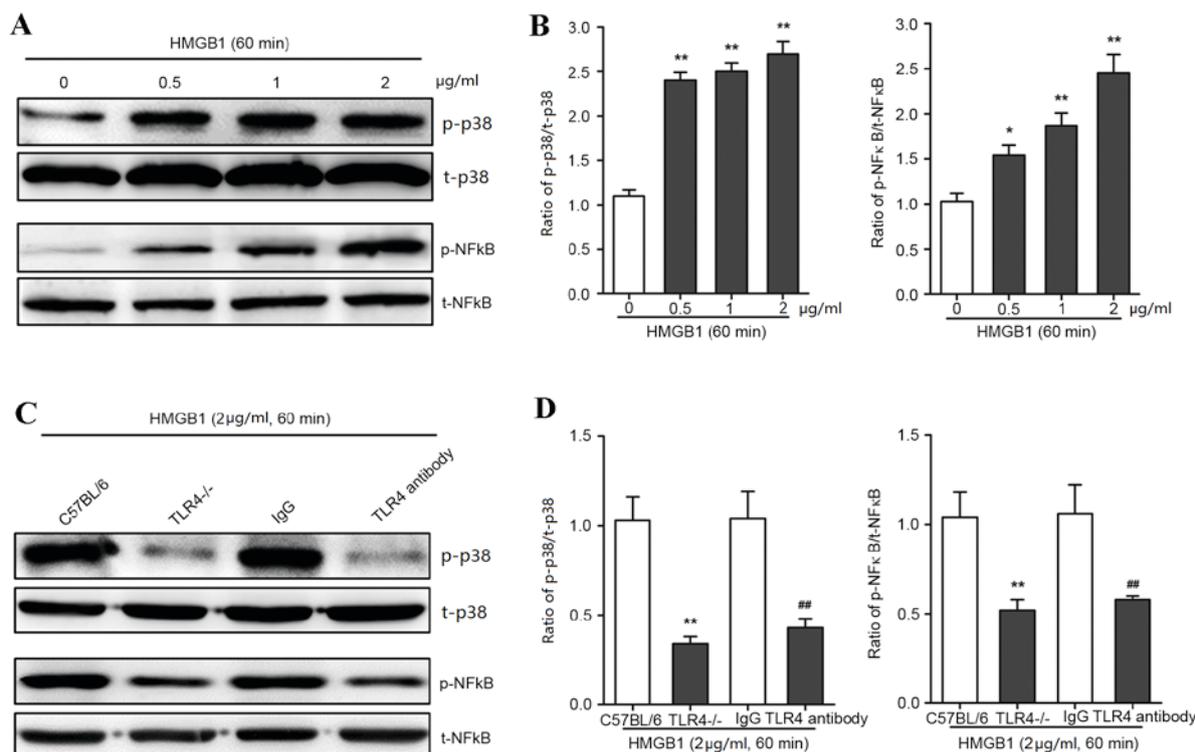
Figure 4. HMGB1 activates p38 and NF-κB in AVICs via TLR4 signaling. Primary AVICs were incubated with 0.5, 1 and 2 µg/ml HMGB1 for 60 min. The group without HMGB1 treatment was used as a control. Primary AVICs derived from TLR4^{-/-} mice or C57BL/6 mice, and C57BL/6-derived AVICs treated with a specific anti-TLR4 antibody (or mouse IgG as the control), were treated with 2 µg/ml HMGB1 for 60 min to determine the activation of p38 or NF-κB. The phosphorylation of p38 and NF-κB was detected by (A and C) western blotting and (B and D) quantified by densitometry in 3 independent experiments. [*]P<0.05 and [**]P<0.01 vs. 0 µg/ml HMGB1 control. The results are expressed as relative units (p38 or NF-κB phosphorylated protein/total protein). [**]P<0.01 vs. the C57BL/6 group; [##]P<0.01 vs. the IgG group. HMGB1, high mobility group box 1; AVICs, aortic valve interstitial cells; p-p38, phosphorylated-p38; t-p38, total-p38; p-NF-κB, phosphorylated-nuclear factor κB; t-NF-κB, total-NF-κB; TLR4, toll-like receptor 4.

activation of TLR4 was then inhibited using TLR4-specific antibodies, as described previously (24). TLR4-specific antibodies significantly inhibited the increase in HMGB1-induced calcification in primary AVICs from C57BL/6 mice, as demonstrated by the significant reduction in ALP activity and alizarin S staining when compared with the mouse IgG control (P<0.01; Fig. 3B and C). The protein expression levels of Runx2, Msx2, BMP2 and OPN in the TRL antibody group were significantly reduced when compared with the mouse IgG control (P<0.01; Fig. 3D and E). These findings demonstrated that HMGB1-induced calcification was significantly attenuated following TLR4 knockout and inhibition, which suggests that AVIC calcification in response to HMGB1stimulation may require TLR4.

HMGB1 activated p38 and NF-κB via TLR4 in AVICs. The authors hypothesized that TLR4 may interact with MAPK in AVICs following exposure to HMGB1. The phosphorylation of p38 was significantly increased following HMGB1 treatment at concentrations of 0, 0.5, 1 and 2 µg/ml for 60 min (P<0.01; Fig. 4A and B). Notably, the phosphorylation of NF-κB, as the downstream effect of p38 phosphorylation, was significantly increased following 60 min incubation with 0, 0.5, 1 and 2 µg/ml HMB1 (P<0.01; Fig. 4A and B). To determine whether the HMGB1-induced activation of p38 MAPK was dependent on TLR4 in AVICs, specific anti-TLR4 antibodies and TLR4^{-/-}-derived AVICs were used to reduce TLR4 expression levels and activity. TLR4

knockout and inhibition significantly decreased p38 and NF-κB activation in AVICs when compared with the control treatments (P<0.01; Fig. 4C and D). These findings suggested that the molecular mechanism underlying HMGB1 treatment may involve TLR4 and p38 MAPK to mediate inflammatory responses.

Discussion

Chronic inflammation is involved in CAVD and may induce AVIC calcification (25). HMGB1, as an inflammatory cytokine, may contribute to chronic inflammatory responses (12-14). The results of the current study demonstrated that the expression of HMGB1 was increased in the calcification region of CAVD aortic valves, which suggests that HMGB1 may contribute to the calcification of AVICs. AVICs were then incubated with different doses of HMGB1, and analysis of ALP activity and alizarin red S staining levels demonstrated a dose-dependent increase. Wang *et al* (26) determined that diabetes accelerated saphenous vein graft calcification by ≥2 years following coronary artery bypass grafting surgery; however, HMGB1 mediated the high-glucose-induced calcification in vascular smooth muscle cells of saphenous veins (24). Lai *et al* (27) reported that HMGB1 was involved in aortic aneurysms induced by calcium chloride in mice. These results demonstrate that HMGB1 may function as a key factor that enhances AVIC calcification. The results of the present study demonstrated

that HMGB1 promoted the expression of Runx2, Msx2, BMP2 and OPN in a dose-dependent manner. Runx2, Msx2, BMP2 and OPN are important genes associated with calcification. Runx2 is an osteogenic and chondrogenic transcription factor regulated by post-translational modifications, sub-nuclear localization, and modulatory protein-protein interactions (28). A previous study determined that the expression level of Runx2 was increased in atherosclerotic calcification and endochondral mineralization programs (29). Hydrogen peroxide was observed to activate osteogenic Runx2 (30) and Msx2/Wnt (31) signaling, thus enhancing mineralization. A previous study indicated that BMP2 increased the secretion of OPN through upregulation of ALP, which led to the degradation of tissue pyrophosphate (32). Runx2 promoted OPN expression in cardiovascular calcification (33). The present study revealed that HMGB1 was expressed in calcified valves, and that HMGB1 treatment induced calcification of AVICs and osteogenic protein expression. Therefore, the present findings emphasized the importance of HMGB1 in the calcification of CAVD.

The results of the present study demonstrated that TLR4, as a receptor of HMGB1, mediated the extent of the inflammatory response and participated in the pathology of CAVD. Zeng *et al* (18,34) determined that AVICs from stenotic valves expressed higher levels of BMP2 in response to TLR4 stimulation and NF-κB activation (17,33). In addition, Deng *et al* (16) demonstrated that adult AVICs exhibit greater inflammatory and osteogenic responses to TLR4 stimulation (34). Together, these results suggest that the HMGB1-induced mineralization of AVICs may be regulated by TLR4. The results of the present study indicated that the calcification region of aortic valves expressed higher levels of TLR4 and HMGB1 when compared with control valves. ALP activity and the level of alizarin red S staining were increased by incubating cells with HMGB1; however, these effects were significantly reduced in TLR4$^{-/-}$ mice and when anti-TLR4 antibodies were used to inhibit TLR4. These findings demonstrate that TLR4 may mediate HMGB1-induced AVIC calcification. Furthermore, the protein expression levels of Runx2, Msx2, BMP2 and OPN were significantly reduced following TLR4 knockout or inhibition. Therefore, TLR4 altered calcification and the expression of a various of osteogenic proteins involved in HMGB1-induced AVIC mineralization.

CAVD is a chronic inflammatory disease where the inflammatory response accelerates calcification (6,7). HMGB1 may induce phosphorylation of p38 and NF-κB, thus activating an inflammatory response via TLR4 (15). The present study incubated AVICs with different doses of HMGB1 for 60 min, and the activation of p38 and NF-κB increased in a dose-dependent manner. Additionally, p38 and NF-κB phosphorylation was inhibited following TLR4 knockout or inhibition. These results demonstrated that TLR4 mediated HMGB1-induced p38 and NF-κB phosphorylation, thereby increasing the inflammatory response in AVICs. In addition, this suggests that the p38/NF-κB signaling pathway may be a promoter of inflammatory calcification that participates in the pathology of CAVD.

In conclusion, the present study demonstrated the expression of HMGB1 and TLR4 in the calcification region of CAVD arterial valves. Using an *in vitro* model it was revealed that this activation was functionally and physically associated with AVIC calcification. The clinical implication of the present study was that deactivation of HMGB1 and TLR4 may effectively attenuate the pathogenesis of CAVD.

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